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Chemistry

Role of subunit interfaces in the allosteric mechanism of hemoglobin

(accessible surface area/close-packing/subunit interactions)

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ABSTRACT **We calculate the surface area buried in subunit interfaces of human deoxyhemoglobin and of horse methemoglobin. A larger surface area is buried in deoxy- than in methemoglobin as a result of tertiary and quaternary structure changes. In both molecules the dimer-dimer interface is close-packed. This implies that hydrophobicity stabilizes the deoxy-structure, the free energy spent in keeping the subunits in a low-affinity conformation being compensated by hydrophobic free energy due to the smaller surface area accessible to solvent.**

Monod, Wyman, and Changeux (1) showed that the formation of "quaternary structure," that is, of stable subunit associations in oligomers, plays an important part in regulating the functional activity of globular proteins. They used hemoglobin as an example, to demonstrate how its quaternary structure gives rise to the cooperativity that distinguishes this tetrameric molecule from single chain oxygen carriers such as myoglobin. The α and β chains of hemoglobin are homologous in sequence and in tertiary structure to myoglobin, yet they interact in such a way that the affinity of one ligand site is increased when another is occupied. This can be explained by protein-mediated interactions between binding sites, or more elegantly, by considering two possible modes of subunit association, one of which would keep the polypeptide chains in a tertiary structure with a low affinity for its ligands (the T, for "tense," state) (1).

The x-ray studies done by Perutz and his group (reviewed by Baldwin in ref. 2) have shown that hemoglobin does have two different quaternary structures: that of methemoglobin, an oxidized form representative of the normal liganded molecule, and that of deoxyhemoglobin. Both have a 2-fold symmetry axis relating like subunits: one type of contact between α and β chains (labeled $\alpha_1\beta_1$) is similar in the two forms, while the other ($\alpha_1\beta_2$ or $\alpha_2\beta_1$) differs. Notably, several charge interactions ("salt bridges") are made by the terminal residues of each chain in the deoxy configuration, which are not present in the met form. The tertiary structure of the chains is less perturbed, and many of the movements occurring within a subunit are related to the position of the heme iron atom relative to the plane of the heme group. This is known to vary with the electronic configuration, and thus the state of coordination, of the metal ion.

The availability of structural data, including atomic coordinates from high-resolution x-ray analysis (3,§), causes this system to be of special interest in a study of the geometry and physical chemistry of allosteric interactions. We have shown (4, 5) that useful information can be derived from the values of the *surface area accessible to solvent* (6) and of the *volume occupied by protein atoms* (7). These quantities are calculated from the atomic coordinates of the protein, and a correlation exists between accessibility to solvent and hydrophobic free energy (8). Therefore, measurements of accessible surface areas yield estimates of the contribution of hydrophobicity to the stability of protein folding (9) and of protein–protein associations (4): for both, hydrophobicity is the largest source of stabilization free energy. On the other hand, specificity is expressed in the close-packing of atoms inside the folded protein or in interfaces between subunits. This is demonstrated by calculating the volume occupied by these atoms, which is found to be the same as in other close-packed structures, such as crystalline amino acids. The $\alpha_1\beta_1$ contact in horse methemoglobin is typical from this point of view (4). The question may, however, be asked whether this is true of the other subunit contacts in hemoglobin, which change when ligands are bound. Here, we present a comparative study of the subunit interfaces in the deoxyhemoglobin and methemoglobin quaternary structures. We show that they are *significantly more favorable in terms of hydrophobic free energy in deoxyhemoglobin*, because its quaternary structure buries a larger surface area than in methemoglobin while maintaining close-packed interfaces. This provides a structural and thermodynamic basis for the allosteric mechanism.

METHODS AND RESULTS

Atomic Coordinates and Molecular Symmetry. The structure of crystalline human deoxyhemoglobin has been established to a resolution of 2.5 Å by Ten Eyck and Arnone (10). Refined atomic coordinates have been published by Fermi (3). Ladner *et al.*‡ have obtained atomic coordinates for horse methemoglobin using 2.0 Å crystallographic data. We submitted both sets of coordinates to a version of "energy refinement" (11) that maintains the 2-fold symmetry of the molecules. This improves in a general way the geometry of nonbonded interactions, at the cost of small changes in the atomic positions [root mean square movements of 0.13 Å in deoxyhemoglobin and 0.17 Å in methemoglobin, compared to the precision estimate of 0.4 Å given by Fermi (3)].

Tertiary and Quaternary Structure Changes. The change in quaternary structure has been described by Muirhead *et al.* (13) on the basis of low resolution electron density maps of deoxy- and methemoglobin. They found that one $\alpha\beta$ dimer rotates relative to the other; the relative movement is much larger than the change affecting the dimers themselves, which could not be seen at this resolution. These were later analyzed by Perutz (14) using crystallographic data at 2.8 Å resolution, and constitute the tertiary structure changes.

We used a least-square procedure of McLauchlan (12) to compare the orientation of the subunits. It determines a rigid-body displacement that superimposes equivalent C_α atoms. The corresponding transformation matrix fixes the orientation. Thus,

‡ *Deceased.*
§ R. C. Ladner, E. J. Heidner, and M. F. Perutz, in preparation.

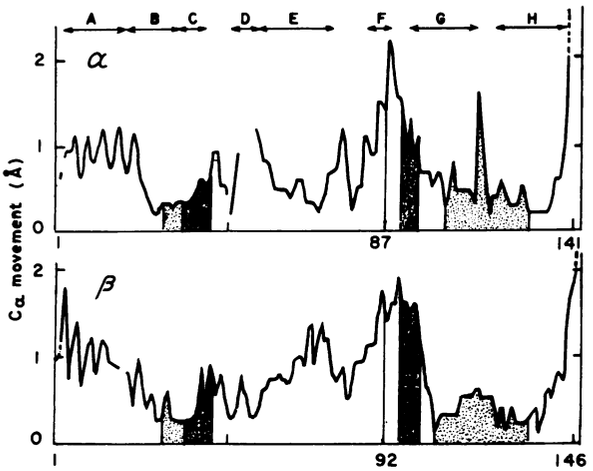

FIG. 1. Tertiary structure changes. The distance between equivalent C_α atoms of deoxy- and methemoglobin after superposition of the $\alpha_1\beta_1$ dimers, is plotted against residue number. The sequences of the α and β chains are aligned to emphasize the similarity of the movements occurring in the two types of chains. Residues 87α and 92β are the heme-linked histidine F8. Residues involved in $\alpha_1\beta_1$ contacts are shaded lightly, those involved in $\alpha_1\beta_2$ contacts more strongly. The positions of the amino- and carboxyl-terminal residues (dashed) are not defined in methemoglobin. The distances expected from random errors in the C_α positions (3) is 0.4 Å.

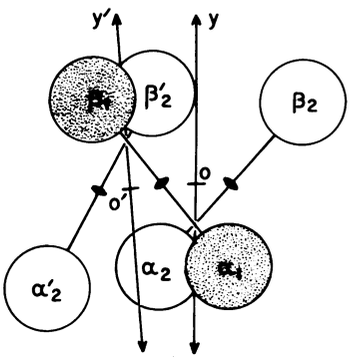

FIG. 2. A schema of the quaternary structure changes. In the coordinate system used for deoxyhemoglobin (13), Oy is the 2-fold symmetry axis relating like subunits, and Ox (normal to the plane of the figure) is parallel to a pseudo 2-fold axis (●) relating α_1 and β_1. When a transformation is applied to the atomic coordinates of methemoglobin in order to superimpose $\alpha_1\beta_1$ with the corresponding deoxyhemoglobin dimer, the position taken by the 2-fold symmetry axis O'y' of the met structure does not coincide with Oy. The symmetry around O'y' generates a new position α'_2 and β'_2 for the second dimer starting from the same position of α_1 and β_1. Using the superposition procedure described in the text, we find that the 2-fold axis Oy and O'y' are nearly coplanar, making an angle of 6.5°, with the molecular centers O and O' 2 Å apart.

we superimposed the deoxy α_1 chain onto the corresponding met chain, and did the same for the β_1 chains; within the precision of the present set of high resolution data, the same transformation matrix was obtained for α_1 and β_1. This confirms that the $\alpha_1\beta_1$ dimer behaves as a rigid body in the conformation change. Residual movements affecting atoms of that dimer after superposition express the tertiary structure change, the amplitude of which is limited since the root mean square residual distance between equivalent C_α position is only 0.9 Å. Fig. 1 shows that many sections of the polypeptide chains are identical in human deoxyhemoglobin and in horsemethemoglobin. As noted by Perutz (14), the tertiary structure change appears to be localized at the corner F and G helices and at the carboxyl-terminal end of both α and β chains. In the FG corner, main chain atoms move by about 1.5 Å; this region includes the heme-linked histidine residue (his F8: 87α and 92β) and is also implicated in the $\alpha_1\beta_2$ contact (see below). The carboxyl-terminal residues make many interchain interactions in deoxyhemoglobin, while they appear to be free in solution in methemoglobin (14, 15). Smaller movements are also seen in the A helix, which is remote from the heme group; however, the position of this helix differs in human and horse deoxyhemoglobins (3), possibly because of their different amino-acid sequences.

In a reference frame linked to the $\alpha_1\beta_1$ dimer, the quaternary structure change can be viewed as a movement of the two-fold symmetry axis relative to $\alpha_1\beta_1$. This generates two different positions for the symmetry-related $\alpha_2\beta_2$ dimer (Fig. 2). In agreement with the results of Muirhead *et al.* (13), $\alpha_2\beta_2$ rotates by about 13° (twice the rotation of the 2-fold axis), around an axis parallel to the pseudo 2-fold axis relating α_1 to β_1. Individual residues move by 3–8 Å.

Surface Area Buried in Subunit Interfaces. The surface area accessible to solvent is defined for given protein atoms as the area of a surface over which a water molecule can be placed, so that it makes van der Waals contact with these atoms without penetrating any other protein atom. We calculate accessible surface area from atomic coordinates by the procedure of Lee and Richards (6), using a computer program of Levitt. The calculation is done for each subunit and for each pair of subunits, $\alpha_1\beta_1$, $\alpha_1\beta_2$, $\alpha_1\alpha_2$, and $\beta_1\beta_2$, in the deoxy- and methemoglobin tetrameric structures. The surface area buried in each contact is then defined as the sum of the accessible surface areas of the subunits minus that of the pair (4). Amino-acid residues whose accessible surface area is less in one pair than in the isolated subunits, constitute the interface of that pair. Because very few residues are involved in more than one interface, the buried surface areas are, to a good approximation, additive. A calculation of the accessible surface area of the tetramer confirms this result.

Table 1 summarizes the results of these calculations. The formation of the tetramer from four subunits decreases the surface area accessible to solvent by about 6000 Å^2. Thus, the interfaces bury one-fifth of the subunits' surface areas, which are close to 7000 Å^2 in accordance with a correlation established previously between molecular weight and accessible surface area of globular proteins (9). In horse methemoglobin, 60% of the area buried in the quaternary structure is found in the $\alpha_1\beta_1$ (and $\alpha_2\beta_2$) interface. This involves about 20 residues on each chain 4). Despite several amino-acid substitutions, the same residues form the $\alpha_1\beta_1$ interface of human deoxyhemoglobin, a result to be expected because no tertiary structure change is seen in this region (Fig. 1) and because the $\alpha_1\beta_1$ dimer moves as a rigid body in the quaternary structure change.

The interface between dimers involves contacts between α_1 and β_2, α_2 and β_1, and between like subunits. These contacts are affected by both tertiary and quaternary structure changes, but not by amino-acid substitutions between human and horse (except at residue 43β). In deoxyhemoglobin, the surface area buried in the $\alpha_1\beta_2$, $\alpha_2\beta_1$, and $\alpha_1\alpha_2$ contacts is 3100 Å^2. It is about 800 Å^2 less in methemoglobin. We see (Fig. 3) that about 11 residues of each chain lose accessible surface area in $\alpha_1\beta_2$ contacts in deoxyhemoglobin, but several of them (Pro 37α, Pro 44α, Arg 141α, Pro 100β, Tyr 145β, and His 146β) do not do so in methemoglobin. The last two residues on the carboxyl-terminal end of the α and β chains contribute 22% of the buried surface area in deoxyhemoglobin, and their release into solution in methemoglobin accounts for one-half of the difference.

Table 1. Surface area buried in contacts

	Human deoxyhemoglobin		Horse methemoglobin	
	Surface area (Å²)	Polar fraction (%)[b]	Surface area (Å²)	Polar fraction (%)
Accessible surface area of subunit[a]				
α	7100	37	6900	37
β	7500	41	7200	40
Surface area buried in contact				
$\alpha_1\beta_1$ or $\alpha_2\beta_2$	1650	37	1750	32
$\alpha_1\beta_2$ or $\alpha_2\beta_1$	1300	40	950	38
$\alpha_1\alpha_2$	500	65	$\leqslant$200[c]	—
$\beta_1\beta_2$	0	—	$\leqslant$200[c]	—
Total buried surface area[d]	6400	40	~5800	35

[a] The accessible surface area of isolated subunits is calculated in the conformation observed in the tetrameric molecules. Values near 7200 Å² are expected on the basis of the molecular weights (9). After differences due to amino-acid substitutions are subtracted, the values obtained for α chains in deoxy- and in methemoglobin are the same to within 3%, and to within 2% for the β chains. This yields an estimate of the precision in determining accessible (or buried) surface areas from two independent crystallographic structure determinations.

[b] Fraction of surface area contributed by nitrogen, oxygen, and, marginally, sulfur atoms.

[c] The calculations based on atomic coordinates provided by Ladner give significant values for the surface buried between like subunits in methemoglobin. However, most of this comes from the amino- and carboxyl-terminal residues for which little density is seen in the electron density map‡, and which are probably free to move in solution.

[d] Contacts between α and β chains occur twice; contacts between like chains, once.

Table 1 also shows that the chemical composition and hydrophobicity of the surfaces involved in contacts between subunits are similar to those of the rest of the protein surface, 32–41% of the area being due to polar atoms in all cases, except for the area of contact between α chains in deoxyhemoglobin. This is much more polar, due to a large contribution of the charged groups carried by the carboxyl-terminal arginine, which forms "salt bridges" across the $\alpha_1\alpha_2$ interface (3, 14).

Interfaces Are Close-Packed. The packing of atoms inside a molecular structure can be analyzed through the volume they occupy (7). The volume occupied by an atom is that of a space-filling polyhedron (Voronoi polyhedron) separating it from all adjacent atoms. We use Richards' method (7) and computer program to calculate the volume occupied by atoms that become buried in contacts between subunits. Then, we compare them with average values of the volume occupied by corresponding atoms in internal residues of known protein structures (9). For 214 atoms buried in the $\alpha_1\beta_2$ and $\alpha_1\alpha_2$ interfaces of deoxyhemoglobin, the sums of expected and observed volumes agree to within 3.8%, and for 144 atoms buried in the $\alpha_1\beta_2$ interface of methemoglobin, to within less than 1%.

The volume occupied by residues inside protein has been shown to be the same as in crystals of small organic compounds, i.e., in crystalline amino acids, which are close-packed. As a consequence, the interfaces between dimers in deoxyhemoglobin, and the $\alpha_1\beta_2$ interface in methemoglobin are also close-packed, as was already observed for the $\alpha_1\beta_1$ interface (4).

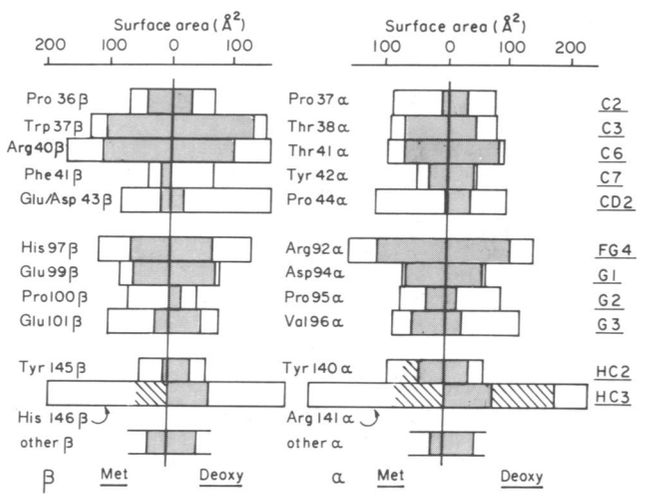

FIG. 3. Surface area of residues involved in the $\alpha_1\beta_2$ contact. Empty bars represent the surface area remaining accessible to solvent in the deoxy- or methemoglobin tetramer. The area buried in the $\alpha_1\beta_2$ interactions is shaded. Residues HC2 and HC3 at the carboxyl-terminal end also lose accessible surface area in contacts between like chains (lined area). Their accessibility to solvent is probably underestimated in methemoglobin, where their position is not fixed.

DISCUSSION

The x-ray analysis of crystalline hemoglobin, done on a large number of different forms of the molecule in the presence or in the absence of ligands, strongly supports a two-state model for its regulatory properties. A quantitative interpretation (2, 19, 20) has been given of the cooperative oxygen binding and of the effects of pH (Bohr effect) and organic phosphates, which are of major physiological importance. The model is based on the hypothesis that the R-state of hemoglobin, like myoglobin and like the dissociated $\alpha\beta$ dimer, has a high affinity for the heme ligands, while the interactions between the two dimers in the T-state keep the subunits in a conformation where they have a low affinity for oxygen or carbon monoxide. Normal deoxyhemoglobin and methemoglobin are representative, respectively, of the T and R structures.

The affinity of the heme group for its ligands depends on the spin-state of the iron. It is normally high-spin when pentacoordinated and low-spin when the sixth coordination valency is occupied by a ligand. Because high-spin iron has a larger ionic radius, ligand removal is coupled with a movement of the iron out of the plane of the heme, as it is found to be deoxyhemoglobin (3, 14, 15). The iron movement is connected with a

change in tertiary structure affecting the FG corner in both the α and β chains (Fig. 1), to which the iron atom is linked via His F8 (87α or 92β). The residues of the FG corner move more than 1 Å away from their subunit, towards the $\alpha_1\beta_2$ interface to which they make a larger contribution in deoxy- than in methemoglobin (Fig. 3).

Because dissociated hemoglobin, like monomeric globins, is in the high-affinity configuration, *the tertiary structure changes observed in the deoxy structure must be energetically unfavorable*. Then, how do the better contacts made between dimers account for this energy difference and, in addition, for the observed stabilization energy of the T structure ($RT \ln L \sim 5$ kcal/mol for $L \sim 35{,}000$ at neutral pH) (2) in the absence of ligand? The charge interactions made in deoxyhemoglobin by amino- and carboxyl-terminal residues have been cited as the source of this energy (14). However, the groups making these "salt bridges" are free to interact with water in the met-structure; moreover, they are still quite accessible to the solvent in deoxyhemoglobin (Fig. 3). Their contribution as a difference can hardly exceed the estimate of Perutz and Ten Eyck (15) (6 kcal/mol for six salt bridges). Similarly, van der Waals contacts made between subunits replace those with water in the dissociated dimers. They cannot favor association unless the interface is close-packed, which is the case both in the deoxy and in the met structures. Though we find, using the present atomic coordinates, that more van der Waals contacts are present in the first, as should be expected from the larger buried surface area, the actual contribution to the free energy of dissociation is certainly small.

On the other hand, a correlation established between surface area accessible to solvent and hydrophobic free energy (8) can be used to demonstrate that the reduction of accessible surface area occurring upon subunit association is the main source of free energy stabilizing protein–protein interactions. For tetrameric hemoglobin, Table 2 shows that this amounts to 55 kcal/mol in the met structure, and about 20 kcal/mol *more* in the deoxy structure, where the surface area buried in the interface between dimers is larger. In other words, *hydrophobicity contributes about 20 kcal/mol of tetramer to the stability of the T structure*. Thus, as much as 5 kcal/mol of subunit can be spent in changing the tertiary structure. Because this free energy is derived from the different surface areas buried between dimers in the R and T structures, blocking the quaternary structure change should also prevent the tertiary structure from changing. This is observed in structural studies done on mutant hemoglobins or in normal hemoglobin after crosslinking (16, 17): only minor atomic movements occur upon reduction of methemoglobin and upon oxidation or CO binding to a deoxy structure. This is especially true of the residues involved in "salt bridges," which make large contributions to the change in buried surface area.

Table 2 also gives estimates of the free energy required to form stable associations between hemoglobin subunits. A minimum value is the free energy released, due to the fewer degrees of translational and rotational freedom of two molecules forming a complex. This can be estimated (4) for free particles *in vacuo* using statistical thermodynamics. We see that the surface area buried in the $\alpha_1\beta_2$ structure is too small to compensate the loss of translational/rotational entropy, leading to dissociation of this dimer, while the larger $\alpha_1\beta_1$ interface provides an excess of hydrophobic free energy, leading to a stable association. An obvious conclusion is that the tetrameric structure is essential in order to stabilize the $\alpha_1\beta_2$ interface, which is responsible for the regulatory properties of hemoglobin. Though the $\alpha_1\beta_1$ interface plays little part in these, dimer formation doubles the hydrophobic contribution to the free energy of dissociation with only a small increase of the translational/rotational term, which varies rather slowly with the size and mass of the molecule. Thus, the balance of hydrophobic and rotational/translational free energies explains the observed stability of the $\alpha_1\beta_1$ dimer and of the tetrameric R and T structures.

Table 2. Free energy of subunit dissociation

	T state	R state		
	Tetramer	Tetramer	$\alpha_1\beta_2$ dimer	$\alpha_1\beta_1$ dimer
Buried surface area (Å^2)	3100[a]	2300[a]	950[b]	1750[b]
Hydrophobic free energy of dissociation (kcal/mol)[c]	75	55	25	45
Translational/rotational free energy of dissociation (kcal/mol)[d]	−30	−30	−27	−27
Free energy of dissociation (observed value, kcal/mol)[e]	⩾14	8	—	⩾11

[a] Surface area buried in dimer-dimer association, that is, in $\alpha_1\beta_2$, $\alpha_2\beta_1$, $\alpha_1\alpha_2$, and $\beta_1\beta_2$ contacts (see Table 1).

[b] Surface area buried in $\alpha_1\beta_1$ or in $\alpha_1\beta_2$ contacts as observed in the tetrameric methemoglobin structure.

[c] 25 cal/mol per A^2 of buried surface area (9).

[d] Calculated from the translational and rotational partition functions of free particles:

$$Z_t = (2\pi mkT)^{3/2}V/h^3; \qquad Z_r = 8\pi^2(2\pi kT)^{3/2}I^{1/2}/h^3$$
$$G = -kT\ln(Z_t{}^N/N!) - kT\ln(Z_r{}^N)$$

[e] $\Delta G = -RT\ln K_D$. The dissociation constant of tetrameric oxyhemoglobin has been measured by Kellet (18); upper limits are given for deoxyhemoglobin (2) and for dissociation of $\alpha_1\beta_1$ into subunits. For $\alpha_1\beta_1$ dimers and for the R tetramer (but not the T tetramer, where the subunit structure is changed), ΔG should be equal to the sum of [c] plus [d] if these are the only major terms controlling association. This is approximately true of the dimer, but the R tetramer is much less stable than expected. This reflects, among other things, the loose structure of the terminal regions which are probably free in solution and so contribute little to the energy of dissociation (see footnote [c] of Table 1).

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